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IN VITRO INHIBITION OF CELL GROWTH OF MOLT-4 MALIGNANT HUMAN T-LYMPHOBLASTS BY COENZYME F_{420}

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Abstract—The inhibitory effect of methanogenic coenzymes on the proliferation of MOLT-4 human malignant T-lymphoblasts was tested. Furthermore the effects of methanogenic coenzymes on dihydrofolate reductase activity (DHFR) from chicken liver have been examined. The results showed that heat-stable extracts of the hydrogenotrophs *Methanobacterium thermoautotrophicum, Methanoculleus thermophilicum* and *Methanogenium tationis* inhibit both proliferation of human T-lymphoblasts and DHFR activity. Heat-stable extract of the methylotroph *Methanosarcina barkeri* showed neither inhibitory nor stimulatory effects in both test systems. The present study proves coenzyme F_{420} to be the active, inhibitory component in methanogenic extracts.

Methanogenic bacteria gain their energy for the biosynthesis of cell components from the reduction of a limited number of C_1-substrates to methane. Hydrogenotrophs are able to grow on H_2/CO_2 or formate, whereas methylotrophs can use methanol, acetate or methylamines as C_1-substrate. Besides the ability to produce methane, methanogenic bacteria are in a number of aspects aberrant from eubacteria [1]. One of these characteristics is the presence of a whole series of unique coenzymes. A coenzyme, which plays a role as one-carbon carrier in the process of methanogenesis is methanopterin [2]. Among various methanogens slight variations exist in the structure of this C_1-carrier. Hydrogenotrophic methanogens generally contain methanopterin, whereas methylotrophic methanogens mostly contain sarcinapterin [3], which has an additional glutamate residue in the side chain. Recently in *Methanogenium tationis* a novel pterin was detected called tatiopterin [4]. Tatiopterin is identical to sarcinapterin, with the exception that it contains an additional aspartic acid in the side chain and a proton instead of a methyl group at the 7-position of the pterin. The structures of these methanogenic pterin derivatives are shown in Fig. 1. *Methanoculleus thermophilicum* contains another pterin derivative, called thermopterin, of which the structure is under investigation. The pterin derivatives in methanogens play a similar role as C_1-carrier as folate derivatives do in eubacteria and eukaryotes.

Another methanogenic coenzyme, which plays a role as electron carrier in methanogenesis and intermediate pathways is coenzyme F_{420} of which the structure is shown in Fig. 1 [5]. Hydrogenotrophic methanogens generally contain coenzyme F_{420} with two glutamate residues (coenzyme F_{420}-2) in the side chain, whereas in methylotrophic methanogens coenzyme F_{420} with four and five glutamate residues (coenzyme F_{420}-4 and F_{420}-5) are present [3]. Coenzyme F_{420} can be considered as a functional analog of NADPH. Both are two-electron carriers.

During the past two decades much work has been done on the development of folic acid antagonists as potential antitumor agents. The classical folic acid antagonist methotrexate (MTX) is one of the most widely used clinical antitumor agents, employed in the treatment of acute lymphoblastic leukemia and other clinical disorders [6]. The basic action of MTX is the inhibition of DHFR. This enzyme catalyses the conversion of dihydrolic acid (H_2F) into tetrahydrofolic acid (H_4F). In this reaction NADPH is used as electron carrier. Depletion of H_4F affects the conversion of deoxyuridylate to deoxythymidylate by thymidylate synthetase, subsequently reducing the intracellular levels of deoxythymidine triphosphate. MTX is intracellularly metabolized to poly(γ-glutamyl) derivatives [7] which are equipotent with MTX as DHFR inhibitors [8] and are retained intracellularly to a greater extent than MTX [9], thus allowing high intracellular concentrations of drug to be attained. Methanogenic pterin derivatives may be considered as analogs of folate polyglutamates in which the polyglutamate chain is replaced by the two pentose units and a phosphate in diester linkage with α-hydroxyglutaric acid.

In this study we report on the effects of heat-stable extracts of various hydrogenotrophic and methylotrophic methanogens on chicken DHFR activity and on proliferation of MOLT-4 human malignant T-lymphoblasts. Furthermore, the effects of methanopterin and coenzyme F_{420} purified from these extracts, were studied in both test systems.

MATERIALS AND METHODS

Materials. MTX (Emtrexate) was purchased from

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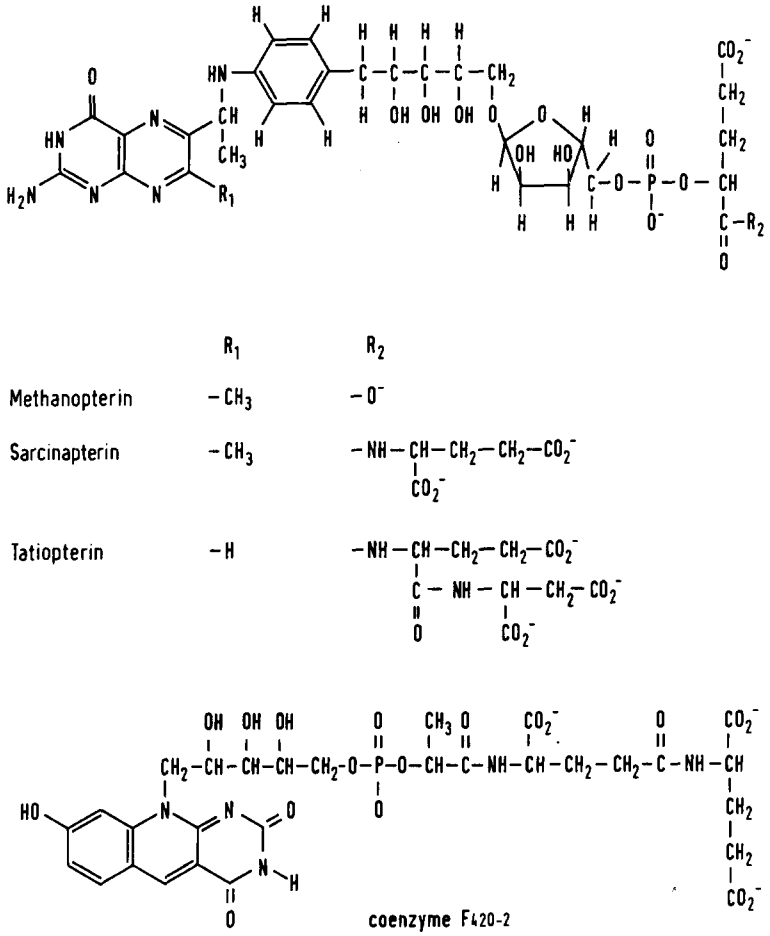

Fig. 1. Structures of methanopterin, sarcinapterin, tatiopterin and coenzyme F_{420}-2.

Pharmachemie (Haarlem, The Netherlands); chicken liver DHFR and H_2F were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); MOLT-4 human malignant T-lymphoblasts from Flow Laboratories (Irvine, U.K.). Gases were obtained from Hoek Loos (Schiedam, The Netherlands) and freed of oxygen by passage over a catalyst: BASF RO-20 at room temperature for hydrogen containing gases and prereduced BASF R3-11 at 150° in all other instances. The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, F.R.G.).

Cultures. Methanogenium tationis (DSM 2702) and *Methanoculleus thermophilicum* (formerly called *Methanogenium thermophilicum* [10]) (DSM 2373) were cultured in a medium described by Zabel *et al.* [11, 12] on H_2/CO_2 (80:20, v/v) in 12-L fermentors with continuous gas flow (6.4 L/min). *Methanobacterium thermoautotrophicum* strain ΔH (DSM 1053) was cultured on H_2/CO_2 according to Schönheit *et al.* [13], *Methanosarcina barkeri* strain MS (DSM 800) was grown as described by Hutten *et al.* [14] and *Methanobacterium bryantii* (DSM 863) was grown on H_2/CO_2 according to Whitman and Wolfe [15].

MOLT-4 cells were grown at 37° in a water-saturated atmosphere containing 5% CO_2 in RPMI medium 1640 Dutch Modification (DM), supplemented with 10% non-dialysed fetal calf serum (v/v), penicillin (100,000 units/L) and streptomycin (100,000 μg/L) (total volume 3 mL) in plastic culture flasks. In order to avoid peroxide formation by light in the presence of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), sodium pyruvate was added to a final concentration of 2 mM [16]. Exponentially growing cells were suspended in 2.7 mL fresh medium at a concentration of 0.33×10^6 cells/mL 24 hr before each experiment. During the experiments glutamine was added every 24 hr (30 μL, final concentration 2 mM) in order to prevent glutamine exhaustion of the medium [17].

MTX, heat-stable extracts of methanogens, and purified coenzyme F_{420} or methanopterin were dissolved in RPMI-DM medium and added as a single dose (in a total volume of 300 μL). An appropriate volume of medium was added to untreated cells. As various methanogens contain different coenzyme concentrations [3], the absorption at 340 nm (A_{340}; which is supposed to be the specific absorption maximum of pterin derivatives) was used as a measure of the concentration of pterin derivatives in heat-stable extracts. Volumes of these extracts were added to give a final A_{340} of 0.6–0.7 in the MOLT-4 assay.

The number of viable cells (Trypan blue exclusion) was counted at each point of time in duplicate in a Bürker-Türk chamber.

Preparation of heat-stable cell extracts. Methanogenic extracts were prepared by adding an equal volume of demineralized water (Milli Q, Millipore, Molsheim, France) to harvested cells and boiling aerobically for 20 min. Cell debris was removed by centrifugation for 30 min at 30,000 *g*. The supernatant was stored at −70° under N_2 atmosphere. Extracts tested in MOLT-4 assays were lyophilized, dissolved in RPMI-DM medium and sterilized over an 0.2 μm filter before use.

Purification of methanopterin and coenzyme F_{420}. Methanopterin was isolated from the hydrogenotroph *Methanobacterium bryantii* since cells of this organism were available in large amounts. Cells (640 g wet wt) were suspended in an equal volume of demineralized water and incubated for 40 min under $N_2/CO_2/O_2$ (80/15/5, by vol.) to convert coenzymes to the oxidized form. From this suspension a cell extract was made by boiling for 40 min. Cell debris was removed by centrifugation at 18,000 *g* for 40 min. The supernatant was adjusted to pH 5.0. Denaturated proteins were removed by centrifugation at 18,000 *g* for 30 min. The supernatant was applied to a DEAE-Sephadex A25 column (22 × 5 cm) equilibrated with 50 mM ammonium acetate buffer, pH 5.0. Subsequently, the column was eluted with this buffer until no more fluorescent compounds were eluted, followed by a 2 L linear gradient of 50–2000 mM ammonium acetate, pH 5.0. Blue fluorescent fractions eluting between 1400 and 1700 mM ammonium acetate, showing an absorption maximum at 340 nm (which is supposed to be characteristic for pterin derivatives) were pooled and applied on a Bonded Phase Octadecylsilane column, (27 × 2.5 cm; particle diameter 40 μm) (Baker) equilibrated with sodium acetate buffer, pH 5.0, containing 2 M NaCl. Subsequently, the column was eluted with a 400 mL linear gradient of 0–50% ethanol in demineralized water. Methanopterin containing fractions, eluting between 26 and 33% ethanol, were lyophilized and dissolved in RPMI-DM medium as described above. A mixture of coenzyme F_{420}-2 (90%) and F_{420}-3 (10%) was purified from *Methanobacterium thermoautotrophicum* essentially according to Eirich *et al.* [5].

UV-visible spectroscopy. UV-visible absorption spectra were recorded on a Hewlett Packard 10833 A spectrophotometer. For determination of methanopterin concentrations the pH was adjusted to 6.0. The molar absorption coefficient at this pH has been reported to be 7.4 $mM^{-1}\,cm^{-1}$ [18]. For determination of coenzyme F_{420} concentrations the pH was adjusted to 8.85. The molar absorption coefficient at this pH is 45.5 $mM^{-1}\,cm^{-1}$ [5].

Dihydrofolate reductase assay. Initial rate assays were performed with a Hitachi U3200 spectrophotometer at 25° under N_2 atmosphere in anaerobic quartz cuvettes using anaerobic solutions. The H_2F-stock solution was stored under N_2 atmosphere at pH 3–4 and −20° in the dark to prevent oxidative degradation. NADPH solutions were made up freshly every day and kept on ice. The rate of enzyme-dependent decrease in the absorbance at 340 nm was followed. The standard mixture for this assay contained 0.15 M KCl in 0.05 M Tris buffer (pH 7.4), 25 μM NADPH and 25 μM H_2F. Conversion of H_2F to H_4F was started by adding 0.016 units DHFR (4.33 μg protein). The total volume was 2 mL. In determining the initial rate the following extinction coefficients were used (at pH 7.0 and 340 nm): NADPH, 6.22 $mM^{-1}\,cm^{-1}$; H_2F, 6.4 $mM^{-1}\,cm^{-1}$ [19]; H_4F, 1.5 $mM^{-1}\,cm^{-1}$ [20]. To test for the effects of methanogenic extracts, methanopterin or coenzyme F_{420} on the reaction, the enzyme was preincubated for 30 min at 25° with these coenzymes before addition to the assay mixture. As in the MOLT-4 assays the A_{340} was used as a measure for the concentration of pterin derivatives. We added methanogenic extracts to the reaction mixture to give an increase in A_{340} of 1.2.

RESULTS

Effects of methanogenic extracts on cell growth and viability compared to the effect of MTX

Figure 2 shows the effects on cell growth and cell viability, respectively, of MOLT-4 cells during incubation with extracts from *Mb. thermoautotrophicum*, *Ms. barkeri*, *Mg. tationis* and *Mc. thermophilicum*. MTX (0.2 μM) was used as reference.

Cell growth stopped immediately after addition of MTX to MOLT-4 cells. Cell viability gradually decreased (from 77% viable cells at 24 hr after addition to 3% viable cells at 72 hr after addition). These results are in good agreement with data published previously [17]. The extract of *Mc. thermophilicum* completely inhibited MOLT-4 cell growth. Cell viability gradually decreased from 37% (at 24 hr) to 14% viable cells (at 72 hr). During incubation with the extract of *Mg. tationis* a slight increase in cell growth was observed until 48 hr, after which cell growth decreased quickly. Cell viability gradually decreased from 74% (at 24 hr) to 21% viable cells (at 72 hr). During incubation with the extract of *Mb. thermoautotrophicum* cell growth increased until the end of the experiment, although with a minor velocity with respect to the control assay (in which culture medium was added instead of heat-stable extract). Cell viability slightly dropped from 92% (at 0 hr) to 73% (at 48 hr) and remained constant until 72 hr after addition. If the concentration of the *Mb. thermoautotrophicum* extract in the medium was increased with a factor three the effect was more pronounced. It was also shown that the extract of *Mb. thermoautotrophicum* had a similar effect on proliferation of L1210 cells *in vitro* (unpublished results). Addition of *Ms. barkeri* extract showed almost no effects on cell growth or cell viability.

Effects of methanogenic extracts on DHFR activity

Table 1 shows the initial velocities of the DHFR reaction if DHFR was preincubated with extracts from various methanogens. The results show a strong inhibition of DHFR activity by the extract of *Mb. thermoautotrophicum*; inhibition of DHFR activity by extracts of *Mc. thermophilicum* and *Mg. tationis*

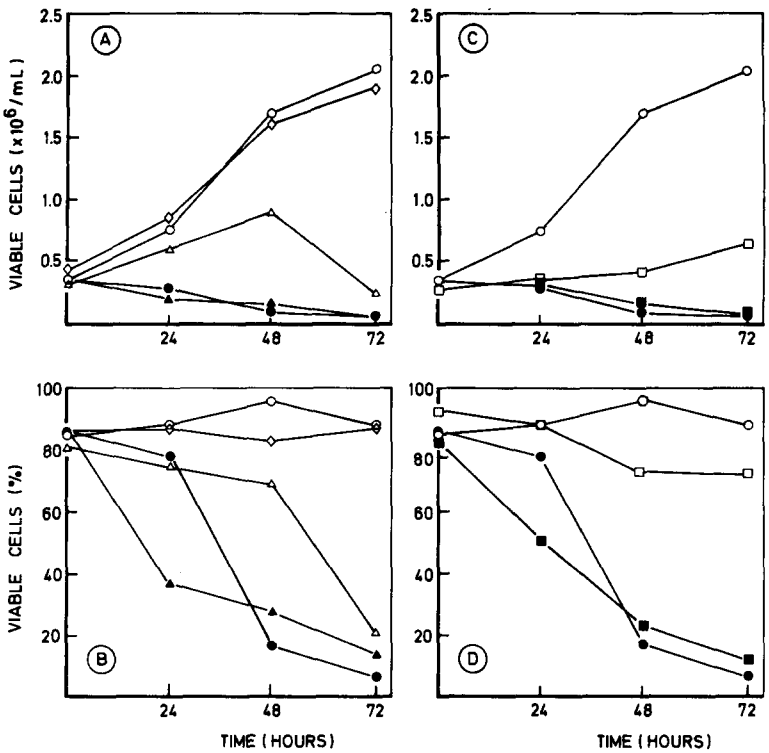

Fig. 2. (a) Effects of MTX and various methanogenic extracts on the growth of MOLT-4 cells. Counts are expressed as the number of viable cells only (Trypan blue exclusion): (○) untreated cells; (●) 0.2 μM MTX; (△) *Mg. tationis*; (▲) *Mc. thermophilicum* and (◊) *Ms. barkeri*. (b) Effects of MTX and various methanogenic extracts on viability of MOLT-4 cells, expressed as percentages of viable plus non-viable cells. See further legend to (a). (c) Effects of different concentrations of extract from *Mb. thermoautotrophicum* on the growth of MOLT-4 cells. Counts are expressed as a number of viable cells only: (○) untreated cells; (●) 0.2 μM MTX; (□) *Mb. thermoautotrophicum*, A_{340} = 0.6; (■) *Mb. thermoautotrophicum*, A_{340} = 1.8. (d) Effects of different concentrations of extract from *Mb. thermoautotrophicum* on viability of MOLT-4 cells, expressed as percentages of viable plus non-viable cells. See further legend to (c).

Table 1. Initial rates of DHFR after preincubation with extracts derived from various methanogens. Methanogenic extracts were added to give an increase in A_{340} of 1.2

Methanogen	Initial rate (μmol H_2F/min.mg protein)
Control*	0.70
Ms. barkeri	0.67
Mg. tationis	0.55
Mg. thermophilicum	0.40
Mb. thermoautotrophicum	0.18

* Control assay in which no methanogenic extract was added.

is somewhat less. The extract of *Ms. barkeri* has almost no effect on DHFR activity.

Effects of methanopterin and coenzyme F_{420} on MOLT-4 growth and DHFR activity

Table 2 shows the effects on growth and viability of MOLT-4 cells during incubation with methanopterin. No significant effects on either cell growth or cell viability were observed with the concentrations used (250–1000 μM). Methanopterin also showed no pronounced effects on chicken liver DHFR activity using 17–170 μM concentrations.

During incubation with 125 μM coenzyme F_{420} no MOLT-4 cell growth was observed during the first 24 hr after addition (Fig. 3), whereafter cell growth continued though with a minor velocity relative to

Table 2. Effects of methanopterin on the growth and viability of MOLT-4 cells

Hours after addition	Concentration of methanopterin (mM) 1.0	0.5	0.25	0.125	0.0
0	0.44*	0.39	0.40	0.44	0.46
	95†	91	94	94	94
24	0.93	1.00	0.98	0.98	0.96
	95	96	98	98	98
48	1.94	1.78	1.57	ND	1.92
	97	95	93	ND	98
72	2.40	2.41	2.39	2.39	2.32
	95	95	92	92	94

* In the first row counts are expressed as the number of viable cells ($\times 10^6$/mL).

† In the second row viability is expressed as percentage of viable plus non-viable cells.

ND, not determined.

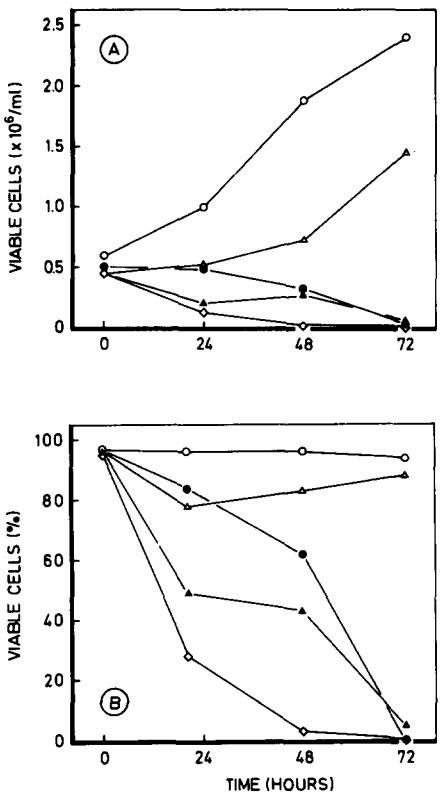

Fig. 3. (a) Effects of MTX and coenzyme F_{420} on the growth of MOLT-4 cells. Counts are expressed as the number of viable cells only: (○) untreated cells; (●) 0.2 μM MTX; (△) 125 μM coenzyme F_{420}; (▲) 250 μM coenzyme F_{420}; (◊) 500 μM coenzyme F_{420}. (b) Effects of MTX and coenzyme F_{420} on viability of MOLT-4 cells, expressed as percentages of viable plus non-viable cells. See further legend to (a).

the control assay. Cell viability decreased to 78% after 24 hr incubation with 125 μM coenzyme F_{420}, whereafter the viability gradually was restored to 87%. During incubation with 250 and 500 μM coenzyme F_{420} a concentration dependent decrease of cell growth and cell viability was observed. At 72 hr after incubation no viable MOLT-4 cells were left.

Coenzyme F_{420} also showed inhibitory effects on chicken liver DHFR activity *in vitro*. Using 60 μM coenzyme F_{420} in the DHFR assay leads to an inhibition on the initial velocity of 45%, whereas 96 μM coenzyme F_{420} leads to an inhibition of 61%. Higher concentrations of coenzyme F_{420} could not be tested in this assay because of too high extinctions, due to a considerable absorbance of coenzyme F_{420} at 340 nm.

DISCUSSION

Studies of Kisliuk [21] indicated that dihydromethanopterin was neither a substrate nor an inhibitor of *Lactobacillus caseï* DHFR at 5×10^{-5} M. The oxidized form of this methanogenic C_1-carrier, methanopterin, has never been tested before as a potential DHFR inhibitor. As most DHFR inhibitors discovered so far are non-reduced compounds, we thought it would be of interest to test the effect of methanopterin and its methanogenic derivatives (sarcinapterin and tatiopterin) on DHFR activity. Heat-stable extracts of various methanogens were used as the source of methanogenic pterin derivatives. In this study we demonstrated that extracts from *Mb. thermoautotrophicum, Mc. thermophilicum* and *Mg. tationis* are potent inhibitors of chicken liver DHFR.

Strikingly, the active inhibitory coenzyme in heat-stable methanogenic extracts proved not to be methanopterin (a folate analog) but coenzyme F_{420}. This coenzyme is able to inhibit both MOLT-4 cell growth and DHFR activity *in vitro*. Furthermore, it is remarkable that extracts of hydrogenotrophic methanogens tested here have an inhibitory effect on MOLT-4 cell growth and DHFR activity, whereas the extract of the methylotroph *Ms. barkeri* has no inhibitory effect on either test system. This may be explained by the fact that *Ms. barkeri* contains mainly coenzyme F_{420}-4 and F_{420}-5, a very small amount of coenzyme F_{420}-3 and no coenzyme F_{420}-2 [22]. The hydrogenotrophic methanogens tested here all contain a considerable amount of coenzyme F_{420}-2 [3]. The inhibitory potential of coenzyme F_{420} derivatives probably is dependent of the length of the polyglutamate side chain. Short chained coenzyme F_{420} derivatives, like coenzyme F_{420}-2 and possibly F_{420}-3 tested here are inhibitory. Purification of various coenzyme F_{420} derivatives, including those even lacking the glutamate residues [5], is needed to determine the most potent derivative and to investigate the nature of the inhibition.

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